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Characterization of *Fusarium graminearum* Mes1 reveals roles in cell-surface organization and virulence

William R. Rittenour, Steven D. Harris *

Department of Plant Pathology and Plant Science Initiative, University of Nebraska-Lincoln, N234 Beadle Center, Lincoln, NE 68588-0660, USA

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Abstract

The surfaces of fungal hyphae are mosaics of carbohydrates and cell-surface proteins. Presently, very little is known about the role of these proteins and their organization at the cell surface. Here, we characterize two *Fusarium graminearum* genes implicated in cell-surface organization, *mes1* (FGSG_06680) and *pls1* (FGSG_08695). Mes1 is a homologue of *mesA*, which is required for the formation of stable polarity axes in *Aspergillus nidulans*. *Pls1* encodes a tetraspanin, which belongs to a class of proteins that have been shown to aggregate in membrane rafts along with integrins and other signaling proteins. Our results indicate that Pls1 is dispensable for saprophytic growth and wheat head infection by *F. graminearum* (a pathogen that does not form appressoria). However, deletion of *mes1* reduces sexual and asexual reproduction, severely perturbs the shape of macroconidia and hyphae, alters the pattern of cell wall deposition and the organization of sterol-rich rafts, and attenuates virulence on wheat heads. Our results provide a basis for identifying determinants of fungal virulence that may localize to specialized domains at the cell surface.

Keywords: *Fusarium graminearum*; Hyphal morphogenesis; Lipid microdomains; MesA; Tetraspanin

1. Introduction

Fusarium head blight (FHB) is a disease of wheat (*Triticum* spp.) and barley (*Hordeum* spp.) caused by several pathogens of the genus *Fusarium*, the most prevalent of which is *Fusarium graminearum* (teleomorph: *Gibberella zeae*). The disease occurs when either macroconidia (asexual spores) or ascospores (sexual spores) are deposited on floral heads, germinate, and invade the floral tissues of host plants (Bushnell et al., 2003). The pathogen displays a short biotrophic phase in the early stages of host invasion, but quickly begins killing plant tissue and switches to a necrotrophic phase. Once infected, wheat and barley heads produce less grain, and residual grain is usually contaminated with *Fusarium* mycotoxins such as deoxynivalenol (DON) and zearlenone. Acceptable thresholds for such mycotoxins are very low, and hence render the grain unmarketable (Dexter and Nowicki, 2003). This reduction in quantity and quality of grain has adversely impacted the agricultural economy of small-grain producing regions. For example, the total economic loss FHB in the 1990s is estimated at $3 billion (Windels, 2000).

The economic impact of FHB highlights the necessity of a viable control strategy. Investigations into the molecular determinants of virulence may identify several targets for limiting the disease, and *F. graminearum* is very suitable for such genetic studies. The genome has been sequenced (Cuomo et al., 2007), an Affymetrix GeneChip is available for microarray analyses (Guldener et al., 2006), and it is tractable to genetic manipulations. Using these molecular tools, several studies have identified molecules and/or molecular pathways necessary for pathogenicity. Deletion of the trichodiene synthase gene *tri5* has demonstrated the importance of DON during host colonization of grain heads (Proctor et al., 1995; Harris et al., 1999; Jansen et al., 2005). Also, several studies have implicated mitogen-activated protein kinases (MAPKs) in plant infection

* Corresponding author. Fax: +1 402 472 3139.
E-mail address: sharri1@unlnotes.unl.edu (S.D. Harris).

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by *F. graminearum* (Hou et al., 2002; Jenczmionka et al., 2003; Urban et al., 2003). More recently, genetic studies have screened mutants generated by randomly inserting hygromycin resistant cassettes throughout the genome using 'restriction enzyme mediated integration' (REMI). This method has identified several factors required for virulence, including methionine synthase, the novel fungal transcription factor Zif1, and a striatin-like protein thought to act as a scaffold for signaling machinery (Seong et al., 2005; Shim et al., 2006).

Genetic studies of virulence thus far have identified several intra-cellular gene products necessary for virulence, but very few cell-surface proteins have been implicated during the infection process. In *Magnaporthe grisea*, the gene *pth11* encodes a transmembrane protein that is essential for pathogenicity, possibly by transducing external cues to internal signaling machinery (DeZwaan et al., 1999; Talbot, 2003). Additionally, G-protein coupled receptors (GPCRs) have been indirectly implicated during the pathogenesis of *M. grisea*. GPCRs transduce external signals to internal heterotrimeric G-proteins to which they are associated (Bolker, 1998). In *M. grisea*, MagB is the component of a heterotrimeric G-protein complex and is essential for pathogenicity, but its associated GPCR has not been identified (Liu and Dean, 1997; Talbot, 2003). New strategies are needed in order to identify cell-surface determinants of virulence/pathogenicity in plant pathogenic fungi. The identification of such proteins may yield highly efficacious antifungal targets that are easily accessible to exogenously applied compounds (Maertens and Boogaerts, 2000; Selitrennikoff and Nakata, 2003).

Cell-surface proteins often aggregate into complexes at the plasma membrane; two such complexes are lipid microdomains and tetraspanin 'webs'. Lipid microdomains are areas in the plasma membrane rich in sphingolipids and sterols, and they have been shown to cluster proteins into complexes involved in protein sorting and signal transduction (Simons and Ikonen, 1997; Alvarez et al., 2007). Recent studies suggest that lipid microdomains may house proteins essential for virulence. In the human pathogen *Cryptococcus neoformans*, virulence determinants phospholipase B1 and a Cu/Zn superoxide dismutase were shown to aggregate into lipid microdomains (Siafakas et al., 2006). Also, the GTPase Ras2 is essential for pathogenicity of *F. graminearum* on wheat and corn silks (Bluhm et al., 2007), and Ras2 has been shown to localize to the intra-cellular side of lipid microdomains in mammalian systems (Parton and Hancock, 2004), though whether or not this occurs in fungi has yet to be determined. Tetraspanins have also been shown to cluster into protein complexes at the surface of animal cells, and these complexes are distinctly different from lipid microdomains (Claas et al., 2001; Levy and Shoham, 2005). Although tetraspanin complexes have not been demonstrated in fungi, the tetraspanin *pls1* is essential for the pathogenicity of *M. grisea*, *Botrytis cinerea*, and *Colletotrichum lindemuthianum* (Clergeot et al., 2001; Gourgues et al., 2004; Veneault-Fourrey et al., 2005). In these appressorium-forming pathogens, tetraspanins seem to be specifically necessary for penetration of the fungus from the appressorium. The importance of tetraspanins in pathogens that do not form an appressorium has yet to be determined.

To determine the significance of these plasma membrane domains in hyphal growth and plant infection by *F. graminearum*, we generated deletion mutants and characterized their growth and pathogenicity phenotypes. The first mutant generated was a deletion of *mes1*, a homologue of *mesA,* which was originally identified as an enhancer of the *sepA1* mutation in *Aspergillus nidulans* (Pearson et al., 2004). MesA is required for the recruitment of SepA (a formin family protein) to the hyphal tip, and hence for the maintenance of stable hyphal polarity. MesA has also been shown to contribute to sterol organization at the hyphal surface of *A. nidulans* (Pearson et al., 2004). In addition to the *mes1* deletion mutant, we also generated a deletion mutant lacking the *pls1* homologue in the *F. graminearum* genome. We show that *mes1* contributes to sexual and asexual reproduction, macroconidia morphology, radial growth, and plant infection by *F. graminearum*, while *pls1* is dispensable for growth, reproduction, and plant infection.

2. Materials and methods

2.1. Strains and culture conditions

All *F. graminearum* strains used in this study were derived from strain PH-1 (NRRL 31084). The Δ*mes1* and Δ*pls1* mutants were generated by transforming strain PH-1 as described below. Stocks were maintained by storing mycelia in 30% (v/v) glycerol solution at −80 °C. Growth rates of strains were tested on solid V8 [10% (v/v) V8 juice, 0.1% $CaCO_3$, 1.5% agar] and YMA (Harris, 2005). Macroconidial stocks for storage and virulence assays were generated by growing strains on either solid YMA or CMC medium (Cappellini and Peterson, 1965) for 7–10 days and liberating the spores in 1.5 ml of sterile-distilled water with a bent glass rod. Solid CMC was preferred over the standard liquid media to prevent macroconidia from germinating before harvest. To assess macroconidia production, 5 ml of liquid CMC was inoculated with 5 μl of a 1×10^5 per ml macroconidial suspension harvested from YMA plates. The cultures then were incubated for 3 days on a rotary shaker at room temperature and the resulting density of macroconidia was counted with a hemacytometer. Macroconidia lengths and widths were measured using differential interference contrast microscopy and IPLab Imaging Software (Scanalytics, Inc.). Conidiophores were imaged after culturing the strains on solid potato dextrose agar (Difco). Biomass was assessed by inoculating 50 ml liquid YMA with 1.3×10^4 per ml macroconidia, followed by incubation on a rotary shaker set at 28 °C and 200 RPM for 3 days. The resulting mycelium was dried at 60 °C for 16 h and the mass was recorded. Sexual crosses were per-